

AMENDMENTS TO THE SPECIFICATION

Please amend the specification as follows:

Please replace the paragraph beginning at page 5, lines 7 through 14 with the following paragraph:

In yet another aspect, the present invention provides methods of inhibiting NF- κ B induction (*e.g.*, IKK α and/or IKK β dependent induction) in a cell by contacting a cell with an effective amount of an anti-inflammatory compound of the present invention, thereby inhibiting NF- κ B induction in a cell. In one embodiment of this invention, such methods utilize anti-inflammatory compounds which include at least one membrane translocation domain. In still another specific embodiment of this invention, the anti-inflammatory ~~[compound-s]~~ compounds utilized in such methods include amino acid sequences comprising the sequences of SEQ ID NO:2, 4, 5, 6, 11, 12, 16, 17 or 18.

Please replace the paragraph beginning at page 6, lines 13 through 16 with the following paragraph:

Figure 2 depicts results from experiments indicating that the first α -helical region of NEMO is required for binding to IKK β . (A) A truncated version of IKK β consisting of only the COOH-terminus from residue V644 to S756 was fused with GST (GST-644-756) and expressed in bacteria. After precipitation by glutathione agarose, GST alone and GST-(644-756) were separated by SDS-PAGE (10%) and the gel was stained with Coomassie blue (left panel). Equal amounts of each protein were used for subsequent GST pull-down analyses. Various NH₂- and COOH-terminal truncations of NEMO were constructed, [³⁵S]-methionine labeled and used for *in vitro* pull down (right panel). Mutants that interacted with GST-(644-756) are indicated (+). None of the mutants interacted with GST alone. (B) Wild-type NEMO and a deletion mutant lacking the first α -helical region (del. α H) were *in vitro* translated (left panel: input) and used for GST pull-down using the proteins shown above (A: left). (C) HeLa cells were transfected with pBIIx-luciferase together with either ~~[peDNA-3]~~ PCDNA-3 (vector) or increasing concentrations of del. α H (0.25, 0.5, 1.0 μ g/ml) for forty-eight hours then treated for four hours

with TNF α (10 ng/ml). Cells were then lysed and NF- κ B activity was measured by luciferase assay.

Please replace the paragraph beginning at page 6, line 27 through page 7, line 17 with the following paragraph:

Figure 3 depicts results from experiments indicating that interaction with NEMO and functional kinase activity requires an IKK α -homologous region of the IKK β COOH-terminus. (A) Truncation mutations of IKK β sequentially omitting the extreme COOH-terminus (1-733), the serine-free region (1-707), the serine rich-domain (1-662) and the α 1-region (1-644) were expressed and labeled by *in vitro* translation and used for GST pull-down by GST-NEMO (Figure 1A). None of the mutants interacted with GST alone. (B) Sequence alignment of the extreme COOH-termini of IKK β and IKK α . The α 2- and glutamate-rich regions are indicated and the six identical amino acids are shaded. (C) Wild-type IKK β and the truncation mutants (1-733 and 1-744) were [35 S]-methionine-labeled (input) and used for *in vitro* pull down with either GST alone or GST-NEMO. (D) HeLa cells were transfected for forty-eight hours with 1 μ g/well of the indicated FLAG-tagged constructs followed by immunoprecipitation using anti-FLAG. The immunoprecipitates were incubated in kinase buffer containing [32 P]-labeled γ ATP for fifteen minutes at 30°C then washed with lysis buffer containing 1% Triton-100. Resulting complexes were separated by SDS-PAGE (10%) and visualized by autoradiography (upper panel). The lower panel is an immunoblot from identical samples demonstrating equivalent amounts of transfected protein in each lane. (E) HeLa cells were transfected for 48 hours with 1 μ g/ml of the indicated constructs or empty vector [~~(pcDNA-3)~~] (PCDNA-3) together with pBIIx-luciferase. NF- κ B activity was determined by luciferase assay. (F) HeLa cells transfected for forty-eight hours with FLAG-tagged versions of either IKK β (wild-type) or IKK β -(1-733) were either untreated (-) or treated for seven minutes (+) with TNF α (10 ng/ml). Following lysis and immunoprecipitation using anti-FLAG, immune-complex kinase assay (upper panels) was performed. Identical samples were immunoprecipitated and immunoblotted with anti-FLAG (lower panels).

Please replace the paragraph beginning at page 7, line 18 through page, line 11 with the following paragraph:

Figure 4 depicts results from experiments indicating that association of NEMO with IKK β and IKK α reveals the NEMO binding domain (NBD) to be six COOH-terminal amino acids. (A) COS cells transiently transfected with vector alone, FLAG-tagged IKK α or IKK β (1 μ g/well) or xpress-tagged NEMO (1 μ g/well) to a total DNA concentration of 2 μ g/well as indicated. Following lysis, immunoprecipitations (IP) were performed using anti-FLAG (M2) and the contents of precipitates visualized by immunoblotting (IB) with either anti-FLAG (M2) or anti-xpress. A portion of pre-IP lysate was immunoblotted with anti-xpress to ensure equivalent levels of NEMO expression in transfected cells. (B) Wild-type IKK α and IKK α -(1-737) were expressed and labeled (input) and used for GST pull-down using GST or GST-NEMO. (C) Full length cDNA encoding human IKK i was obtained by RT-PCR from HeLa cell mRNA using the [~~ExpandTM Long Template PCR System~~] EXPANDTM LONG TEMPLATE PCR SYSTEM (Boehringer Mannheim), the forward primer (5'-CTAGTCGAATTCACCATGCAGAGCACAGCCAATTAC) (SEQ ID NO: 22) and the reverse primer (3'-CTAGTCTCTAGATTAGACATCAGGAGGTGCTGG) (SEQ ID NO: 23) and cloned into the *EcoRI* and *XbaI* sites of [~~peDNA-3~~] PCDNA 3. GST pull-down analysis was performed using [³⁵S]-methionine-labeled IKK α , IKK β and IKK i . (D) A deletion mutant of IKK β lacking the NBD (del.NBD) was [³⁵S]-methionine-labeled (input) and used for GST pull down analysis. (E) A Fauchere-Pliska hydrophobicity plot of the COOH-terminus (N721-S756) of human IKK β was generated using MacVectorTM [\square] (version 6.5.3) software. The NBD (L737-L742) is boxed. (F) COS cells were transfected for forty-eight hours with a total of 2 μ g DNA/well of either vector alone, vector plus NEMO-FLAG or NEMO-FLAG plus xpress-tagged versions of IKK β -(1-744) containing point mutations within the NBD as indicated. Following lysis and immunoprecipitation using anti-FLAG (M2), immunoblot analysis was performed with either anti-FLAG or anti-xpress. The level of expressed protein in pre-IP lysate was determined by immunoblotting with anti-xpress (lower panel). (G) HeLa cells were transiently transfected for forty-eight hours with the indicated constructs together with pBIIX-luciferase and NF κ B activity in lysate was measured by luciferase assay.

Please replace the paragraph beginning at page 8, line 12 through page 9, line 2 with the following paragraph:

Figure 5 depicts results from experiments indicating that a cell-permeable peptide spanning the IKK β NBD inhibits the IKK β /NEMO interaction, TNF α -induced NF- κ B activation and NF- κ B-dependent gene expression. (A) GST-pull-down analysis was performed using either GST-NEMO-*in vitro* translated IKK β (upper panel) or GST-IKK β -(644-756)-*in vitro* translated NEMO (lower panel). The assay was performed in the absence (no peptide) or presence of increasing concentrations (125, 250, 500 or 1000 μ M) of either mutant (MUT) or wild-type (WT) NBD peptide. (B) HeLa cells were incubated with either peptide (200 μ M) for the times indicated. Following lysis, the IKK complex was immunoprecipitated using anti-NEMO and the resulting immunoblot probed with anti-IKK β . (C) HeLa cells were transfected for forty-eight hours with pBIIX-luciferase then incubated for two hours in the absence (control) or presence of mutant or wild-type NBD peptide (100 and 200 μ M of each). Subsequently the cells were either treated with TNF α (10 ng/ml) as indicated (left panel) or left untreated (right panel) for a further four hours after which NF- κ B activation was measured by luciferase assay. (D) HeLa cells were incubated for three hours with increasing concentrations (50, 100 or 200 μ M) of each peptide followed by treatment for fifteen minutes with TNF α (10 ng/ml) as indicated (+). Following lysis, nuclear extracts were made and 10 μ g of protein from each sample was used for EMSA using a specific [32 P]-labeled κ B-site probe. (E) Primary HUVEC were pre-incubated for two hours with of wild-type (left) or mutant (right) NBD peptides (100 μ M) then stimulated with TNF α (10 ng/ml) for a further six hours. Control cells received no peptide. Cells were stained with either anti-E-selectin (H4/18) or a non-binding control antibody (K16/16) and expression was measured by FACS (~~FACSort~~ FACSORT, Becton Dickinson). The profiles show E-selectin staining in the absence (shaded) and presence (solid line) of TNF α and control antibody staining under the same conditions (dashed line, no TNF α ; dotted line, TNF α).

Please replace the paragraph beginning at page 9, lines 15 through 24 with the following paragraph:

Figure 8 depicts the results of a mutational analysis of D738 within the NEMO binding domain (NBD) of human IKK β . (A) The aspartic acid residue at position 738 of IKK β was substituted with either alanine, asparagine or glutamic acid using PCR-mutagenesis. (B) The IKK β (D738) mutants shown in A were ³⁵S-methionine-labeled by *in vitro* transcription and translation then used for GST pull-down analysis using GST-NEMO as previously described. (C) Hela cells were transiently transfected using the [~~Fugene6~~] FUGENE 6 transfection method with the NF- κ B-dependent reporter construct pBIIx-luciferase together with either [~~pcDNA-3~~] PCDAN-3, IKK β or the D738 mutants described above (A). After 48 hours, the cells were lysed and luciferase activity was determined as previously described.

Please replace the paragraph beginning at page 9, line 25 through page 15 through 24 with the following paragraph:

Figure 9 depicts the results of a mutational analysis of W739 and W741 within the NBD of human IKK β . (A) The tryptophan residues at positions 739 and 741 of IKK β were substituted with alanine, phenylalanine, tyrosine or arginine using PCR-mutagenesis. (B) COS cells were transiently transfected with either vector alone [~~(pcDNA-3.1-xpress)]~~ (PCDAN-3.1-XPRESS), IKK β , W739A, W739F or W739Y together with FLAG-tagged NEMO as shown. After 48 hours, the cells were lysed and complexes were immunoprecipitated (IP) using anti-FLAG (M2)-coupled agarose beads. Prior to immunoprecipitation a portion of each lysate (5%) was retained for analysis (pre-IP). Proteins in samples were separated by SDS-PAGE (10%) and analyzed by immunoblotting (IB) using antibodies recognizing either FLAG (M2) or xpress. The upper two panels show xpress-tagged IKK β and the lower panel shows FLAG-tagged NEMO. (C and D) COS cells were transiently transfected with the plasmids shown followed by immunoprecipitation and immunoblot analysis as described in B. (C and D) Hela cells were transiently transfected with pBIIx-luciferase together with the plasmids shown and after 48 hours luciferase activity in lysates was determined.

Please replace the paragraph beginning at page 10, lines 15 through 22 with the following paragraph:

Figure 11 depicts the results of a mutational analysis of the IKK α NBD. (A) Each of the residues that comprise the NBD of IKK α (L738 to L743) were substituted with alanine by PCR-mutagenesis. COS cells were transiently transfected with NEMO-FLAG together with either vector alone (~~pcDNA-3.1-xpress~~) (PCDNA-3.1-EXPRESS) or xpress-tagged versions of IKK α and the NBD mutants as shown. Immunoprecipitation and immunoblot analysis of the IKK α -NEMO complexes was performed as described in Fig.2B. (B) Hela cells were transiently transfected with pBIIx-luciferase together with the plasmids shown and after 48 hours luciferase activity in lysates was determined.

Please replace the paragraph beginning at page 40, lines 8 through 23 with the following paragraph:

All sub-cloning and mutagenesis of full length cDNA clones of IKK α and IKK β was performed by PCR using cloned Pfu DNA-polymerase (~~Stratagene~~) (STRATAGENE). The wild-type and mutated IKK β cDNA were inserted into the *KpnI* and *NotI* restriction sites of [~~pcDNA-3~~] PCDNA-3 or [~~pcDNA-3.1-xpress~~] PCDNA-3.1-XPRESS (Invitrogen) and all IKK α cDNAs were inserted into the *EcoRI* and *XhoI* sites of the same vectors. FLAG-tagged versions of both kinases were constructed by subcloning into pFLAG-CMV-2 (Sigma). For GST-IKK β - (644-756), the PCR fragment was inserted into the *EcoRI* and *XhoI* sites of pGEX-4T1 (Pharmacia). Full length cDNA encoding human NEMO was obtained by reverse transcriptase (RT)-PCR from HeLa cell mRNA using the [~~ExpandTM Long Template PCR System~~] EXPANDTM LONG TEMPLATE PCR SYSTEM (Boehringer Mannheim) and the primer pair (5'-ATAGACGAATTCAATAGGCACCTCTGGAAG) (SEQ ID NO: 20) and (3'-TAGGACCTCGAGCTACTCAATGCACTCCATG) (SEQ ID NO: 21). The resulting PCR fragment was inserted into the *EcoRI* and *XhoI* sites of [~~pcDNA-3~~] PCDNA-3 or [~~pcDNA-s.1-xpress~~] PCDNA-S.1-XPRESS. All subsequent NEMO mutants were constructed by PCR using

Pfu DNA-polymerase. GST-NEMO was constructed by sub-cloning the full-length cDNA into the *EcoRI* and *XhoI* sites of pGEX-4T1.

Please replace the paragraph beginning at page 41, lines 5 through 14 with the following paragraph:

The effects of IKK β -(644-756) on IL-1 β - and TNF α -induced NF- κ B activation by transiently transfecting HeLa cells with the mutant together with an NF- κ B-dependent reporter plasmid (pBIIX-luciferase) was tested next (Kopp & Ghosh, (1994) Science 265, 956-959). For transfection studies, HeLa and COS cells were seeded into either twenty-four well (1×10^5 cells/well) or six well (5×10^5 cells/well) plates and grown for twenty-four hours before transfection of DNA with [~~Fugene6~~] FUGENE6(Roche) according to the manufacturer's protocol. Cells in twenty-four well and six well trays received a total of 1 μ g or 2 μ g of DNA respectively. After forty-eight hours cells were lysed with TNT (200 mM NaCl, 20 mM Tris-pH 8.0, 1% Triton-100) and the lysate were used for either immunoprecipitation or luciferase assay (Primage Luciferase Assay System).

Please replace the paragraph beginning at page 48, lines 7 through 14 with the following paragraph:

The sequences of the two NBD peptides used in this study were [~~DRQIKIWFQNRRMKWKK~~]TALDWSWLQTE [DRQIKIWFQNRRMKWKK]TALDWSWLQTE (wild-type) (SEQ ID NO:18) and [~~DRQIKIWFQNRRMKWKK~~]TALDASALQTE [DRQIKIWFQNRRMKWKK]TALDASALQTE (mutant) (SEQ ID NO:19). The *antennapedia* homeodomain sequence (Derossi *et al.*, (1994) J. Biol. Chem. 269, 10444-10450; U.S. Patent No. 5,888,762; U.S. Patent No. 6,015,787; U.S. Patent No. 6,080,724) is bracketed and the positions of the W to A mutations are underlined. Both peptides were dissolved in DMSO to a stock concentration of 20 mM. For all experiments DMSO alone controls were no different from no peptide controls.

Please replace the paragraph beginning at page 19, line 31 through page 20, line 12 with the following paragraph:

Cmpd. No.

- 1 H-RRMKWKKTALDWSWLQTE-NH₂ (SEQ ID NO: 161);
- 2 H-YGRKKRRQRRRTALDWSWLQTE-NH₂ (SEQ ID NO: 162);
- 3 H-rrrrrrTALDWSWLQTE-NH₂ (SEQ ID NO: 163);
- 4 H-YARKARRQARRTALDWSWLQTE-NH₂ (SEQ ID NO: 164);
- 5 H-YARAARRAARRTALDWSWLQTE-NH₂ (SEQ ID NO: 165);
- 6 H-RRMKWKKLDWSWL-NH₂ (SEQ ID NO: 166);
- 7 H-rrmkwkkLDWSWL-NH₂ (SEQ ID NO: 167);
- 8 H-rrrrrrLDWSWL-NH₂ (SEQ ID NO: 168);
- 9 H-YARAARRAARRLDWSWL-NH₂ (SEQ ID NO: 169);
- 10 H-yaraarraarrLDWSWL-NH₂ (SEQ ID NO: 170); and
- 11 H-YGRKKRRQRRRLDWSWL- NH₂ (SEQ ID NO: 171).